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Fructans with Varying Degree of Polymerization Enhance the Selective Growth of *Bifidobacterium animalis* subsp. *lactis* BB-12 in the Human Gut Microbiome In Vitro

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Abstract: Synbiotics aim to improve gastrointestinal health by combining pre- and probiotics. This study evaluated combinations of *Bifidobacterium animalis* subsp. *lactis* BB-12 with seven fructans: oligofructoses (OF1-OF2; low degree of polymerization (DP)), inulins (IN1-IN2-IN3; high DP) and OF/IN mixtures (OF/IN1-OF/IN2). During monoculture incubations, all fructans were fermented by BB-12 as followed from increased BB-12 numbers and increased acetate and lactate concentrations, with most pronounced fermentation for low DP fructans (OF1-OF2). Further, short-term colonic incubations for three human donors revealed that also in presence of a complex microbiota, all fructans (particularly OF1) consistently selectively enhanced the growth of BB-12. While each fructan as such already increased *Bifidobacteriaceae* numbers with 0.94–1.26 log(cells/mL), BB-12 co-supplementation additionally increased *Bifidobacteriaceae* with 0.17–0.46 log(cells/mL). Further, when co-supplemented with fructans, BB-12 decreased *Enterobacteriaceae* numbers (significant except for IN1-IN3). At metabolic level, all fructans decreased pH due to increased acetate and lactate production, while OF/IN2-IN1-IN2-IN3 also stimulated propionate and butyrate production. BB-12 co-supplementation further increased propionate and butyrate for OF/IN2-IN3 and IN1-IN2, respectively. Overall, combinations of BB-12 with fructans are promising synbiotic concepts, likely due to intracellular consumption of low DP-fructans by BB-12 (either present in starting product or released upon fermentation by indigenous microbes), thereby enhancing effects of the co-administered fructan.

Keywords: prebiotic; synbiotic; microbiota; colon; intestine; oligofructose; inulin; *Bifidobacteria*; *Faecalibacterium*; *Blautia*

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1. Introduction

During the past decade, pre-, pro- and synbiotics have been extensively studied with the aim to improve gastrointestinal health. While prebiotics are defined as non-digestible substrates that are selectively utilized by the colonic microbiota, thereby beneficially affecting hosts' health [1], probiotics are defined as live microorganisms conferring a health benefit on the host when administered in adequate amounts [2]. A synbiotic formulation combines both concepts aiming to provide complementary or even synergistic effects in comparison with administration of the individual components [3]. Numerous clinical benefits have been associated with synbiotic supplementation, including reduction of intestinal complaints in patients with gastrointestinal disorders [4,5], alleviation of atopic

dermatitis [6], reduced colon cancer risk [7], improvement of serum lipid levels and supporting weight loss in obese individuals [8,9]. A crucial remark regarding synbiotics is that when a prebiotic substrate would be combined with a randomly selected probiotic, this prebiotic would not necessarily support the growth of the added probiotic [10]. Therefore, it is of importance that the prebiotic supports growth of the co-administered probiotic, thus resulting in more predictable and beneficial outcomes of the prebiotic [10,11].

Fructan-type carbohydrates have been widely as prebiotic component in synbiotic formulations [12]. They occur naturally in many plant-based foods, such as chicory, asparagus and leek, and consist of β-(2,1)-linked fructose molecules with a terminal glucose moiety. Fructans are classified by their degree of polymerization (DP), with molecules with a DP ranging from 2 to 10 being classified as oligofructose (OF), while long-chain fuctans are termed inulin (IN) which is characterized by a DP $\geq$ 10 up to a DP of 60 [13]. Fructan-type carbohydrates escape digestion in the upper gastrointestinal tract and reach the colon where they are fermented by the indigenous microbiota, including health-related *Bifidobacterium* sp. [14]. A commercially available probiotic belonging to the *Bifidobacteriaceae* family that is extensively used in functional foods and dietary supplements is *Bifidobacterium animalis* subsp. *lactis* BB-12. Several health benefits have been attributed to BB-12 intake, including hypocholesterolemic effects [15], improvement of bowel function [16], protection against diarrhea [17] and immunomodulation [18]. As BB-12 is able to ferment fructan-type carbohydrates [19], it has a potential to be combined with fructans as a synbiotic combination. Previously, Mueller et al. demonstrated in BB-12 monocultures that fructan structure affects its fermentation by BB-12 [20]. Therefore, the effectiveness of a fructan-based synbiotic with BB-12 might highly depend on the structure of the selected fructan.

Next, when assessing the potential of a prebiotic to support growth of a probiotic, it is important to consider that the human colon is colonized by a dense microbial community that has as its key function the fermentation of both host and diet-derived carbohydrates [21]. The human colon microbiota thus has a large potential to ferment prebiotics such as fructans. When evaluating the potential fermentation of a prebiotic by a probiotic strain, it is thus important to not only address this research question in monocultures of the probiotic but also to assess this in presence of a (simulated) colon microbiota. Furthermore, given the existence of interindividual differences in microbiome composition among human individuals, mainly caused by genetic background and diet [22], interactions between the probiotic and the indigenous microbiota can differ among donors as for instance demonstrated by the interindividual differences in microbiome modulation upon fructan treatment [23–25]. Furthermore, also colonization of probiotics such as BB-12 could depend on interindividual differences as shown by Matto et al. [26].

The present study aimed to identify which fructan-type carbohydrates supported growth of the probiotic strain BB-12 most optimally, both in BB-12 monocultures as well as during short-term colonic incubations in presence of a mixed microbiota derived from multiple healthy human donors. The study was conducted in three stages (Figure 1). First, potential fermentation of seven different fructan-type carbohydrates by BB-12 was investigated in monoculture incubations with focus on growth of BB-12 (as detected via qPCR) and its resulting metabolic activity (experiment 1). Then, a screening of six human donors was performed with the aim to select three appropriate donors for the final experiment. This screening involved in vitro incubations to: (i) confirm intrinsic capacity of BB-12 to grow in presence of sterile fecal suspension of a given donor, as well as (ii) to assess the diversity of the overall metabolic activity of the fecal microbiota of each donor (experiment 2) Finally, short-term colonic incubations were performed to assess the synbiotic potential of the fructan-type carbohydrates combined with BB-12 in presence of the complex microbiota derived from three selected human donors (experiment 3). During this final experiment, microbial composition was assessed using a novel technique, i.e., quantitative 16S-targeted Illumina sequencing allowing to obtain quantitative insights in microbiome modulation at high phylogenetic resolution [27].

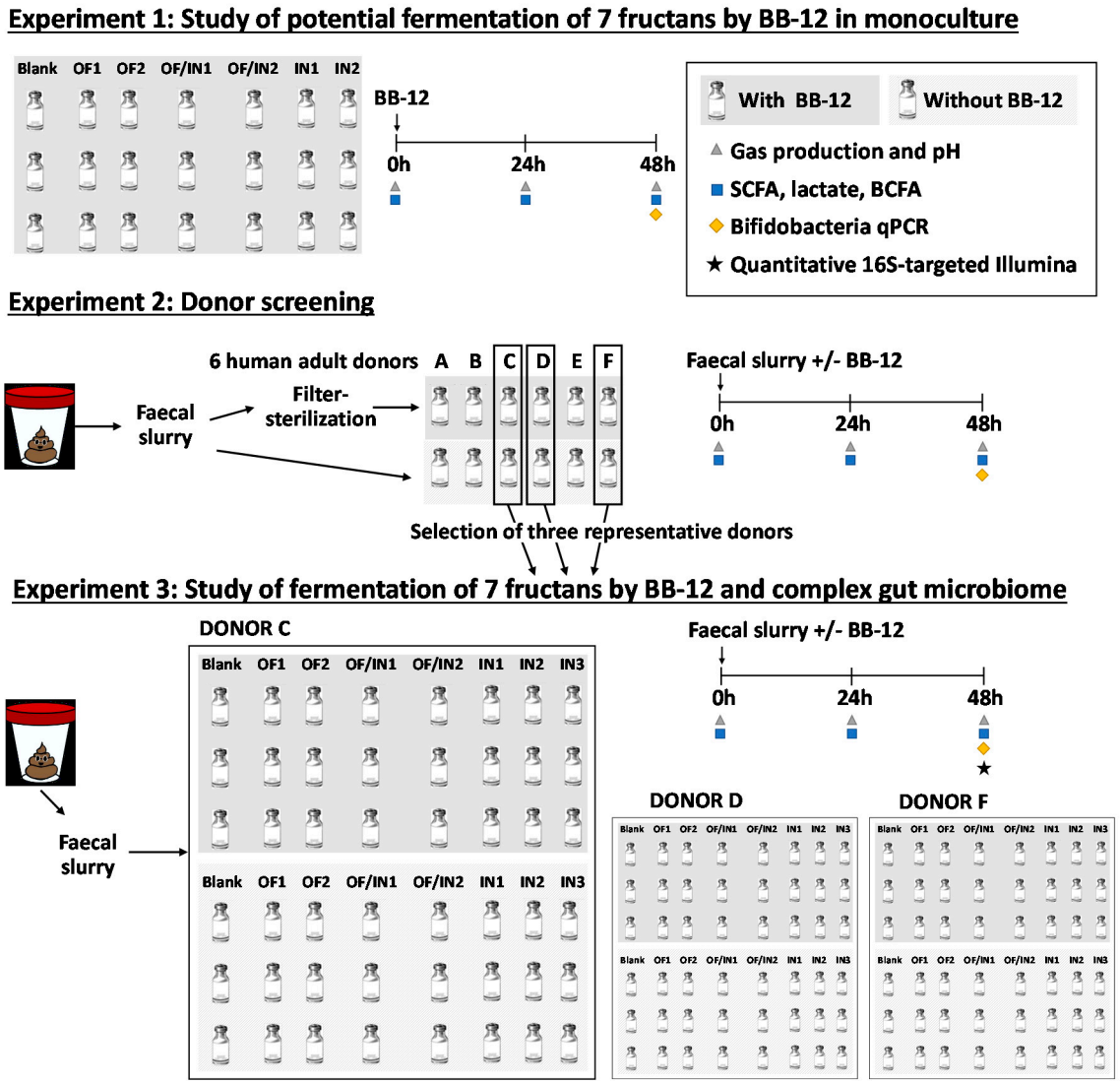

Figure 1. Schematic representation of the in vitro approach to investigate the synbiotic potential of fructan-type carbohydrates and BB-12. First, potential fermentation of seven fructan-type carbohydrates by BB-12 was investigated in monoculture incubations (experiment 1). In experiment 2, a donor screening was performed for six human faecal donor samples. In experiment 3, short-term colonic incubations were performed to characterize the synbiotic potential of the fructan-type carbohydrates combined with BB-12 and complex microbiota of three selected human donors. Samples were collected to evaluate the effect of the test products on microbial metbaolic activity (pH, gas production, SCFA, lactate and bCFA) and community composition (Bifidobacteria qPCR and quantitative 16S-targeted Illumina sequencing). BB-12 = Bifidobacterium animalis subsp. lactis; OF = oligofructose; IN = inulin; SCFA = short-chain fatty acids; bCFA = branched-chain fatty acids; qPCR = quantiative polymerase-chain reaction.

2. Materials and Methods

2.1. Chemicals and Test Product

All chemicals were obtained from Sigma-Aldrich (Overijse, Belgium) unless stated otherwise. *Bifidobacterium animalis* subsp. *lactis* BB-12 strain (Chr. Hansen, Hoersholm, Denmark) was supplied by PepsiCo, Inc. (Moscow, Russia). Seven fructan fibers were obtained from their respective suppliers as explained in Table 1.

Table 1. Overview of the seven fructans tested, including the product code used in the study, the commercial name of the product, the supplier, the purity of the fructan fibers (%), the simple sugar content (%) and the average degree of polymerization (DP). OF = oligofructose; IN = inulin.

Code	Commercial Name	Supplier	Purity Fructan	Sugars [1]	DP
OF1	Frutalose® OFP	Sensus, The Netherlands	89	8	4
OF2	Orafti®P95	Beneo, Belgium	93.2–97.5	2.5–6.8	4–5
OF/IN1	Fibrulose® F97	Cosucra, Belgium	97 ± 2	3 ± 2	5.5
OF/IN2	Orafti® Synergy1	Beneo, Belgium	92 ± 2	8 ± 2	14.5
IN1	Fibruline® Instant	Cosucra, Belgium	>90	<10	9
IN2	Orafti® GR	Beneo, Belgium	>90	<10	10
IN3	Frutafit® IQ	Sensus, The Netherlands	>90	<10	8–13

[1] including fructose, glucose and sucrose.

2.2. Strain Preservation and Quality Control

The provided probiotic strain was grown in liquid culture medium (reinforced clostridial medium—RCM) under anaerobic conditions for 24 h at 37 °C. A subculture was made on a selective and differentiating solid growth medium ((transgalactosylated oligosaccharide agar, containing 12.5 mg/L mupirocin) and the strain was grown for a period of 96 h at 37 °C. A second subculture was made by picking up a colony and growing in RCM under anaerobic conditions for 24 h at 37 °C. The obtained culture was aliquoted and cryopreserved at −80 °C by mixing equal volumes of the culture and a pre-reduced 40% glycerol suspension. A sample of the preserved bacterial culture was subjected to DNA extraction (performed according to Boon et al. [28] with minor modifications as reported by Duysburgh et al. [29]) and send out to LGC Genomics GmbH (Berlin, Germany) for 16S rRNA gene sequencing using the primers and PCR conditions as described by Kok et al. [30]. The obtained sequence was searched against a curated 16S rRNA gene dataset of the ribosomal database project (RDP), using the Seqmatch tool, to confirm strain identity. Further, the cryopreserved strain was grown in a standardized fashion under optimal conditions (RCM medium inoculated at 1%, 37 °C, anaerobic conditions) prior to inoculation in each experiment. The optimal incubation period for growth of the preserved strain was determined at 16 h.

2.3. Fermentation of Fructans by BB-12 in Monoculture during Short-Term Colonic Incubations (Experiment 1)

To evaluate the synbiotic potential of combining the BB-12 test strain with each of the seven fructans, first, a short-term colonic experiment was performed in the absence of indigenous colon microbiota. Briefly, each of the seven fructans were dissolved in water, filtered through a 0.22 µm filter and dosed aseptically to reactors containing sterile carbohydrate-depleted nutritional medium (5.2 g/L K_2HPO_4, 16.3 g/L KH_2PO_4, 2.0 g/L $NaHCO_3$ (Chem-lab NV, Zedelgem, Belgium), 2.0 g/L yeast extract, 2.0 g/L peptone (Oxoid, Aalst, Belgium), 1.0 g/L mucin (Carl Roth, Karlsruhe, Germany), 0.5 g/L L-cysteine and 2.0 mL/L Tween 80; pH = 6.5) in order to reach a concentration of 5 g/L of each fructan at the start of the incubation. A dose corresponding with 10^9 CFU of the BB-12 strain was co-administered, reaching a concentration 1.5×10^7 CFU/mL at start of the incubation. A sterile anaerobic fecal suspension was prepared from a freshly collected fecal sample of a healthy human donor as described by Moens et al. [31] and inoculated at 10% (*v/v*) into the reactors to provide the BB-12 strain with necessary co-factors to perform substrate breakdown. A blank incubation was included, where BB-12 strain was administered without co-supplementation of fructans. All reactors were anaerobically incubated at 37 °C for 48 h under continuous mixing (90 rpm). All experiments were performed in biological triplicate.

2.4. Donor Screening (Experiment 2)

A donor screening was performed using two types of short-term colonic incubations: (1) incubations inoculated with sterile anaerobic faecal suspension of each donor in the presence of the BB-12 strain, and (2) incubations inoculated with non-sterile anaerobic faecal suspension of each donor in the absence of the BB-12 strain. At the start of the short-term colonic incubations, a carbohydrate-depleted nutritional medium (similar as used during experiment 1) and starch (2 g/L) were added to each of the reactors. A dose corresponding with 10^9 CFU of the BB-12 strain was added to the sterile reactors only (test condition (1)). Sterile and non-sterile anaerobic faecal slurries were prepared from freshly collected faeces of six healthy human donors and inoculated at 10% (*v/v*) into the respective reactors of test condition (1) and (2). Informed consent was retrieved for each of the donors to use the fecal samples for the study according to the ethical approval with Belgian registered number B670201836585. Each incubation was performed in single repetition and incubated for a period of 48 h at 37 °C, under shaking (90 rpm) and anaerobic conditions.

2.5. Fermentation of Fructans by BB-12 and Complex Microbiota during Short-Term Colonic Incubations (Experiment 3)

At the start of the short-term colonic incubations, each of the fructans were dosed to a carbohydrate-depleted nutritional medium (similar as used during experiment 1) to obtain a final concentration of 5 g/L for each fructan. The BB-12 strain and complex microbiota were dosed to the reactors. Complex microbiota were obtained from the three selected healthy adult donors from experiment 2 and added to the reactors as an anaerobic faecal suspension corresponding to 10% (*v/v*). A dose corresponding with 10^9 CFU of the BB-12 strain was co-administered, resulting in a concentration 1.5×10^7 CFU/mL at start of the incubation. Two types of controls were included, i.e., incubations without the addition of the BB-12 strain and fructans, and control incubations where the BB-12 strain was administered without co-supplementation of fructans. All reactors were anaerobically incubated at 37 °C for a period of 48 h under continuous mixing (90 rpm) and performed in biological triplicate.

2.6. Analysis of Microbial Metabolic Activity

Samples were collected after 0 h, 6 h, 24 h and 48 h of incubation from each reactor in experiment 1, 2 and 3 for determination of microbial metabolic activity. Measurement of gas production was conducted using a pressure meter (Hand-held pressure indicator CPH6200; Wika, Echt, The Netherlands), while pH values were assessed with a Senseline pH meter F410 (ProSense, Oosterhout, The Netherlands). Short-chain fatty acid (SCFA) concentrations, including acetate, propionate, butyrate and branched SCFA (bCFA; sum of isobutyrate, isovalerate and isocaproate), were determined using the method of De Weirdt et al. [32]. Analysis of lactate levels was performed using a commercially available enzymatic assay kit (R-Biopharm, Darmstadt, Germany) according to manufacturer's instructions.

2.7. Analysis of Microbial Community Composition

During experiment 1 and 2, samples were collected after 0 h and 48 h of incubation from each reactor for determination of bifidobacterial growth through quantitative polymerase chain reaction (qPCR), while during experiment 3 samples collected after 48 h of incubation were subjected to quantitative 16S-targeted Illumina sequencing. DNA was isolated from pelleted bacterial cells originating from 1 mL sample as described by Boon et al. [28] with minor modifications [29].

qPCR analysis for *Bifidobacterium* spp. was performed on a QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in technical triplicate and outliers with more than 1 CT difference were removed from the dataset. The qPCR assay for *Bifidobacterium* spp. was performed as previously reported in Rinttilä et al. [33].

Microbial community profiling during experiment 3 was performed using quantitative 16S-targeted Illumina sequencing analysis (LGC Genomics GmbH) as described by Van den Abbeele et al. [34]. Briefly, results obtained from the Illumina Miseq platform with v3 chemistry were presented as proportional values versus the total amount of sequences within each sample, after which data was combined with total bacterial cell count to obtain quantitative abundances of the different taxonomic entities (phylum, family and OTU level) inside the reactors. Assessment of the total bacterial population was done by flow cytometric analysis on a BD FACSverse (BDBiosciences, Erembodegem, Belgium), followed by analyzation of absolute cell counts using the FlowJo software, version 10.5.2.

2.8. Data and Statistical Analysis

For data within donors the average ± SD was reported, while for data averaged over the three donors the average ± SEM was calculated. With respect to microbial metabolic markers as well as qPCR data, regular 2-sided t-tests were applied for comparisons within a given donor, while paired 2-sided t-tests were applied for comparisons over the donors. In order to correct for multiplicity, the Benjamini-Hochberg false discovery rate (FDR) was applied (with FDR = 0.10) [35]. For statistical analysis of the quantitative 16S-targeted Illumina sequencing data, a value below the limit of quantification (LOQ) was equaled to the LOQ. Then, upon log-transformation of the absolute values (to make data normally distributed), regular 2-sided t-tests were applied for comparisons within a given donor, while paired 2-sided t-test were applied for comparisons over the three donors. Again, the Benjamini-Hochberg false discovery rate (FDR) was applied (with FDR = 0.10). All calculations were carried out via Excel, while figures were prepared in the GraphPad Prism v8.4.2 software.

As a remark, to establish an overall LOQ for quantitative 16S-targeted Illumina sequencing data, first, 1 read was divided by the total amount of reads in each sample, followed by multiplication with the bacterial cell count detected via flow cytometry. This allowed to obtain a LOQ for each sample individually. Then, the minimal LOQ was calculated within each triplicate to identify the lowest value that could possibly be detected for a given condition. Then, over conditions (run in triplicate), the maximal value was determined which provided the overall LOQ for the entire dataset. The overall LOQ corresponded to 5.90 log(cells/mL). Finally, the average within triplicates was calculated and when being below the overall LOQ, the value was reported as below LOQ, even if it was above the LOQ of the individual sample.

3. Results

3.1. Fermentation of Fructan-Type Carbohydrates by BB-12 in Monoculture (Experiment 1)

Potential fermentation of seven fructan-type carbohydrates by BB-12 was investigated in monoculture incubations with focus on endpoints related to microbial metabolic activity and growth of BB-12. During a first series of experiments, in which no sterile fecal suspension was co-administered, BB-12 did not ferment any of the fructans (data not shown). While still no growth was observed in the blank that included sterile fecal suspension (indicating absence of fermentation of the nutrients in the background medium by BB-12), each of the seven fructan-type carbohydrates was fermented by BB-12 (in presence of sterile fecal suspension), as observed by the significant reduction of pH, stimulation of acetate and lactate levels and increased numbers of BB-12 (Figure 2).

Fermentation by BB-12 differed between the fructan-type carbohydrates based on their degree of polymerization. Strongest metabolic effects were observed upon supplementation of oligofructoses (OF1 and OF2), resulting in final levels of approximately 30 mM acetate and 4.5 mM lactate, mainly by enhanced production during the 6–24 h time interval. In contrast, supplementation of the three long-chain inulins (IN1, IN2 and IN3) resulted in similar, though less pronounced, effects, with an approximate increase of 7 mM acetate and 0.5 mM lactate after 48 h of incubation. Treatment with OF/IN1 and OF/IN2 exerted intermediate effects. Furthermore, propionate, butyrate, bCFA and gas were not produced

in the synbiotic incubations (data not shown), which is in line with the inability of BB-12 to produce gas and these metabolites, thus confirming sterility of the faecal suspensions.

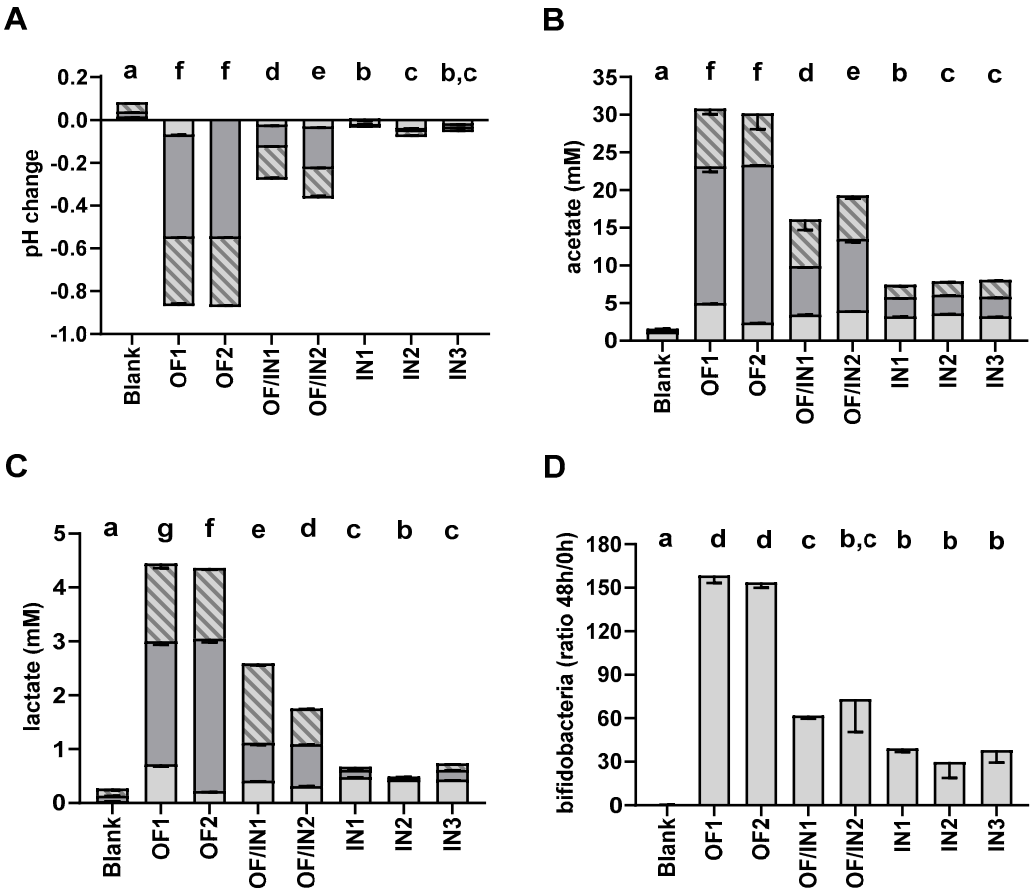

Figure 2. Metabolic activity of BB-12 upon dosing fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) versus a blank incubation in monocultures. Bars represents the average changes (±SD) in pH (**A**), acetate ((**B**); mM) and lactate ((**C**); mM) levels between 0 and 6 h (light grey), 6 h and 24 h (dark grey) and 24 h and 48 h (stripes) as well as changes in *Bifidobacterium* levels ((**D**); expressed as the ratio of their absolute abundance as measured via qPCR at the end versus the beginning of the incubation (48 h/0 h)) (n = 3). Conditions that have no letter in common are statistically significantly different from one another ($p < 0.05$). BB-12 = *Bifidobacterium animalis* subsp. *lactis;* SD = standard deviation; OF = oligofructose; IN = inulin.

In consistency with aforementioned metabolic data, growth of the BB-12 strain was observed for all fructan-type carbohydrates. Strongest stimulatory effects (approximately factor 150 increase versus start of the incubation) were observed for the oligofructoses (OF1 and OF2), while mildest stimulatory effects were observed with the long-chain inulins (IN1, IN2 and IN3). Finally, OF/IN1 and OF/IN2 resulted in intermediate enrichments.

3.2. Donor Screening (Experiment 2)

The donor screening of six donors consisted of two types of colonic incubations with starch as main carbon source, i.e., (1) incubations with sterile fecal suspension of a given donor in presence of BB-12 to confirm engraftment of BB-12 in background suspension derived from a certain donor, and (2) incubations with non-sterile fecal suspension of each donor in absence of BB-12 to investigate the metabolic activity of the fecal microbiota derived from each donor as such. To focus on consistent observations, the averages over the six donors are presented in Figure 3, while individual data are reported in Figures S1 and S2.

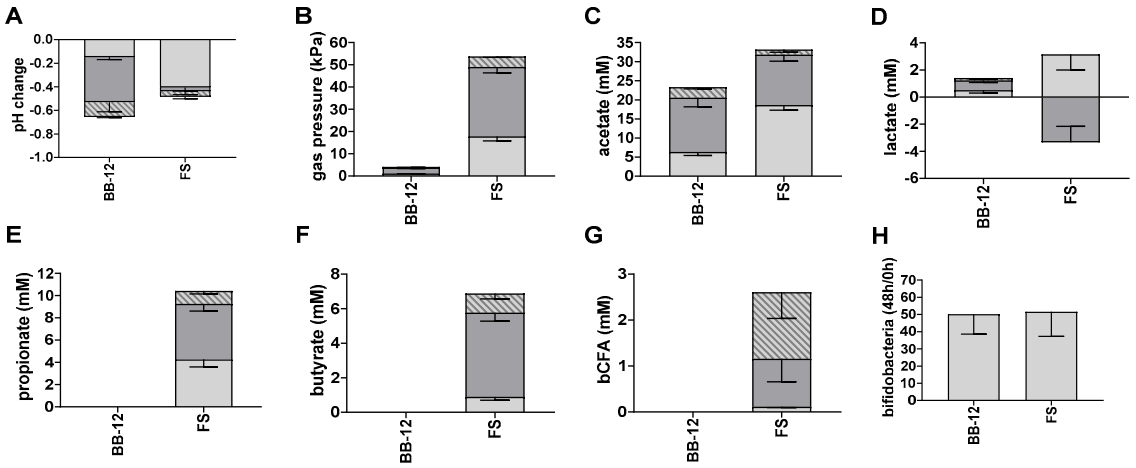

Figure 3. Metabolic activity of BB-12 in presence of sterile fecal suspension of six donors ('BB-12′) and of the fecal microbiota of the six same donors as such ('FS') upon dosing starch as carbon source. Bars represents the average changes (±SEM) in pH (**A**), gas production (**B**), acetate ((**C**); mM), lactate ((**D**); mM), propionate ((**E**); mM), butyrate ((**F**); mM) and bCFA ((**G**); mM) levels between 0 and 6 h (light grey), 6 h and 24 h (dark grey) and 24 h and 48 h (stripes) as well as changes in *Bifidobacterium* levels ((**H**); expressed as the ratio of their absolute abundance as measured via qPCR at the end versus the beginning of the incubation (48 h/0 h)) (n = 6). BB-12 = *Bifidobacterium animalis* subsp. *lactis;* SEM = standard error of means.

First, BB-12 grew in the fecal background of all donors tested as shown by a reduction in pH and increase of acetate, lactate and *Bifidobacterium* spp. levels after 48 h of incubation (Figure 3). As was observed during experiment 1, propionate, butyrate and bCFA were not produced in the probiotic incubations, confirming sterility of the fecal suspensions. Supplementation of BB-12 generated the highest acetate concentrations for donors D and E, while the lowest acetate levels were observed for donor B (Figure S1). This was in line with the observations of bifidobacterial growth: while BB-12 was enriched for all donors, the strongest and mildest increases were observed for donors D/E and donor B, respectively. Overall, acetate was mainly produced during the 6–24 h timeframe upon supplementing BB-12. Further, BB-12 produced similar, yet low lactate quantities for all donors in the incubations with sterile fecal suspension.

Next, the metabolic profiles of the fecal microbiota of all six donors revealed consistently reduced pH together with increased gas production and enhanced production of acetate, lactate, propionate, butyrate and bCFA (Figure 3). Upon its initial production, lactate was subsequently consumed. While some minor interindividual differences were observed, all donors revealed similar metabolic responses (Figure S2). Furthermore, enrichment of the Bifidobacterium population was observed for all donors during the colonic experiments, with strongest effects observed for donors C and F, while lowest enrichment was observed for donor E.

Altogether, these data indicate that all donors could be selected for the final experiments. In the end, three representative donors with sufficient interindividual differences were selected, i.e., donors C (highest butyrate production), donor D (highest acetate production) and donor E (lowest bCFA production).

3.3. Fermentation of Fructan-Type Carbohydrates by BB-12 and Complex Microbiota (Experiment 3)

To characterize the synbiotic potential of the fructan-type carbohydrates in combination with BB-12 in presence of a complex microbiota, short-term colonic incubations were performed using the fecal inocula of the three selected donors. Upon performing 16 S-targeted Illumina sequencing coupled with flowcytometry on the samples collected after 48 h of incubation to obtain absolute numbers, an OTU5 related to *Bifidobacterium animalis*, i.e., species to which BB-12 belongs to, was detected in all reactors to which BB-12 was dosed (72 independent reactors), while not being detected in any of the reactors to

which BB-12 was not dosed (again 72 independent reactors; data not shown). Therefore, although 16S-targeted Illumina sequencing cannot be considered as a species or strain-specific detection method in general, detection of OTU5 was considered to reflect the abundance of BB-12 for this very specific experimental setup. This allowed to conclude that upon its administration to the complex microbiota, mild enrichment of BB-12 (as reflected by OTU5) was observed in the probiotic incubations (blank) of donors C (Figure 4A), D (Figure 4B) and F (Figure 4C). The selective growth of BB-12 was strongly boosted upon its co-administration with each of the seven fructan-type carbohydrates, as seen by the significant enrichment compared to the blank incubations for all fructans and for all donors tested. When averaged over the three donors (Figure 4D), the strongest growth of BB-12 was observed for OF1, followed by OF2 and OF/IN1, OF/IN2, while the mildest growth of BB-12 was observed for IN1.

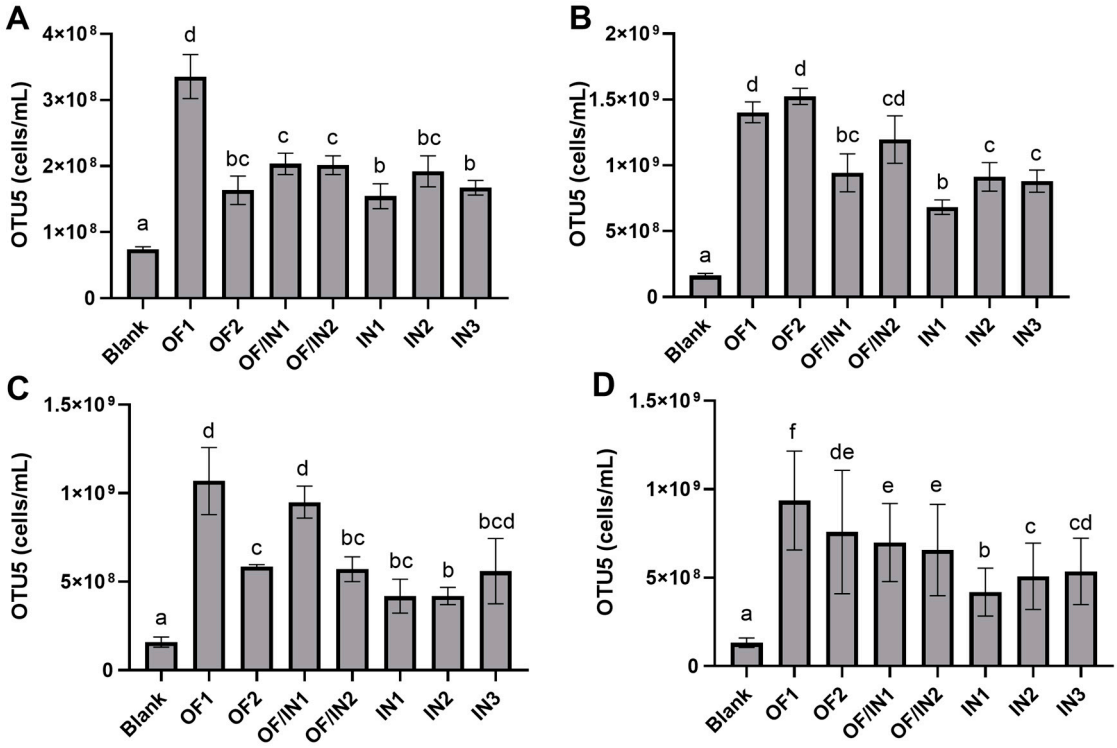

Figure 4. Selective growth of OTU5 (related to *Bifidobacterium animalis* and upon data analysis reflecting the levels of BB-12) in presence of a complex microbiota upon dosing BB-12 and fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) versus a blank incubation. Bars represent the average absolute levels (cells/mL) of OTU5 after 48 h of colonic incubation for donors C (**A**), D (**B**) and F (**C**) together with the averages over the three donors (**D**) ($n = 3$ for each donor). Results are expressed as average ± SD for the individual donors and average ± SEM for the average over the donors. Conditions that have no letter in common are statistically significantly different from one another ($p < 0.05$). OTU = operational taxonomic unit; BB-12 = *Bifidobacterium animalis* subsp. *lactis;* OF = oligofructose; IN = inulin; SD = standard deviation; SEM = standard error of means.

While addition of BB-12 as such did not alter the pH, acetate, lactate, butyrate or bCFA levels compared to the blank (Table 2), minor, yet significant increases in gas and propionate production were observed, mainly due to increases for donor C (Table S1). In contrast, marked and significant metabolic changes were observed upon dosing the fructan-type carbohydrates, including reduction of pH values, increased gas production, stimulation of acetate and lactate levels and decreased bCFA production (Table 2). Similar effects were observed upon supplementation of OF1, OF2 and OF/IN1, which were characterized by a more pronounced increase of lactate levels. Treatment with OF/IN2, IN1, IN2 and IN3 on the other hand significantly increased propionate and butyrate levels, coinciding with

stronger increases in gas production. Some additional changes were observed when BB-12 was co-administered with fructans. Dosing BB-12 together with OF2 further enhanced acetate levels, while BB-12 treatment together with OF2, OF/IN2 and IN3 further stimulated propionate production. Finally, BB-12 enhanced butyrate levels upon co-administration with OF/IN1, IN1 and IN2.

Table 2. Changes in metabolic activity upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of three human donors (C, D and F). The values represent average differences over the three donors versus the respective reference (for fructans, the reference was the untreated blank, while for conditions with BB-12 addition this was the respective condition where BB-12 was not dosed). The data represent the average changes between 0–48 h (except for the intermediate metabolite lactate where it is the maximal value measured throughout the incubation). Significant changes are indicated in bold (n = 9; 3 values for each of the 3 donors).

Endpoint	Blank	OF1		OF2		OF/IN1		OF/IN2		IN1		IN2		IN3	
	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12
pH	0.00	**−0.93**	0.07	**−0.97**	0.02	**−0.86**	0.01	**−0.78**	0.05	**−0.68**	−0.03	**−0.80**	0.00	**−0.77**	0.01
Gas (kPa)	**2.4**	**28.1**	2.9	**21.5**	**4.6**	**30.6**	3.3	**48.9**	−1.3	**45.9**	1.0	**45.0**	0.7	**43.8**	2.2
Acetate (mM)	0.6	**20.4**	−0.2	**20.1**	**4.3**	**22.5**	1.0	**23.6**	−0.3	**24.6**	−2.8	**26.3**	**−4.2**	**24.5**	1.0
Lactate (mM)	0.1	**15.1**	**−0.9**	**15.1**	−1.2	**14.1**	**−1.2**	**7.8**	0.3	**7.6**	0.5	**8.1**	0.2	**9.2**	−0.2
Propionate (mM)	**0.4**	0.2	0.4	0.6	**0.9**	1.0	0.1	**4.3**	**1.2**	**5.5**	−0.1	**4.4**	0.3	**3.4**	**1.2**
Butyrate (mM)	0.1	2.4	0.6	0.2	0.3	1.9	**1.1**	**5.5**	1.4	**5.9**	**1.4**	**4.2**	**1.9**	**5.6**	−0.1
bCFA (mM)	0.1	**−2.8**	0.0	**−2.8**	0.0	**−2.8**	0.1	**−2.7**	0.0	**−2.7**	0.1	**−2.8**	0.1	**−2.7**	0.1

Finally, quantitative 16S-targeted Illumina sequencing was performed to obtain insights in microbial composition changes upon treatment with BB-12 and/or the different fructans. Data are presented at phylum (Table 3 for average of the donors and Table S2 shows the data per individual donor) and family level (Table 4 for average of the donors and Tables S3–S5 for the data at per individual donor), while also the 20 most abundant OTUs are reported (Table 5 for average of the donors and Tables S6–S8 for the data per individual donor).

Table 3. Changes of microbial abundances at phylum level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of three human donors (C, D and F). The values represent average differences versus the respective reference (for fructans, the reference was the untreated blank, while for conditions with BB-12 addition this was the respective condition where BB-12 was not dosed) at the end of the colonic incubation over the three donors. Significant changes are indicated in bold (n = 9; 3 values for each of the 3 donors).

Phylum	Blank	OF1		OF2		OF/IN1		OF/IN2		IN1		IN2		IN3	
	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12
Actinobacteria	**0.19**	**0.63**	**0.30**	**0.67**	**0.17**	**0.64**	**0.22**	**0.64**	**0.26**	**0.63**	**0.22**	**0.58**	**0.30**	**0.65**	**0.27**
Bacteroidetes	−0.01	**−0.54**	0.02	**−0.52**	0.09	**−0.45**	0.03	−0.09	**0.15**	0.09	0.02	−0.05	0.02	−0.17	0.13
Firmicutes	0.01	0.10	−0.03	**0.19**	**−0.11**	0.01	−0.05	**0.20**	**0.12**	**0.23**	0.06	0.15	0.06	0.11	0.04
Proteobacteria	0.00	−0.04	−0.09	−0.07	−0.14	0.04	−0.08	0.08	−0.08	0.05	−0.03	0.04	−0.05	0.07	−0.04
Verrucomicrobia	0.14	−0.10	−0.02	0.02	0.06	0.04	−0.06	−0.05	0.17	0.05	0.03	0.02	0.16	0.09	0.08

Table 4. Changes of microbial abundances at family level (log(cells/mL)) as detected via quantitative 16S−targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of three human donors (C, D and F). The values represent average differences versus the respective reference (for fructans, the reference was the untreated blank, while for conditions with BB-12 addition this was the respective condition where BB-12 was not dosed) at the end of the colonic incubation over the three donors. Significant changes are indicated in bold (n = 9; 3 values for each of the 3 donors).

Phylum	Family	Blank	OF1		OF2		OF/IN1		OF/IN2		IN1		IN2		IN3	
		BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12
Actinobacteria	*Bifidobacteriaceae*	**0.54**	**1.20**	**0.29**	**1.26**	**0.17**	**1.05**	**0.35**	**1.06**	**0.38**	**0.99**	**0.41**	**0.94**	**0.46**	**0.97**	**0.46**
	Coriobacteriaceae	−0.10	0.14	0.32	0.16	0.20	0.30	0.09	0.15	0.08	−0.01	**0.25**	−0.02	**0.27**	0.16	0.21
	Eggerthellaceae	−0.02	0.12	**0.28**	0.08	0.16	**0.39**	0.05	**0.46**	0.01	**0.42**	0.04	**0.41**	0.08	**0.51**	0.06
Bacteroidetes	*Bacteroidaceae*	−0.03	**−0.82**	0.01	**−0.82**	0.09	**−0.58**	0.04	−0.13	**0.16**	0.14	0.03	0.01	0.00	−0.15	0.11
	Marinifilaceae	0.10	−0.18	0.03	−0.33	0.24	−0.05	−0.09	−0.13	0.13	−0.23	−0.06	−0.09	0.16	−0.06	0.02
	Muribaculaceae	0.09	0.10	−0.05	0.19	−0.04	0.00	0.04	0.08	−0.23	−0.05	−0.04	0.06	0.12	0.08	0.01
	Prevotellaceae	−0.02	−0.06	0.07	0.28	0.08	−0.08	−0.01	0.03	−0.10	−0.18	0.00	0.12	−0.03	−0.24	0.18
	Rikenellaceae	−0.07	0.18	0.01	0.21	0.01	0.15	0.00	0.03	0.11	0.03	0.09	0.07	0.07	0.09	0.11
	Tannerellaceae	−0.03	**−1.16**	0.01	**−1.21**	0.05	**−0.94**	−0.06	**−0.90**	**0.23**	**−0.93**	0.08	**−0.88**	**0.14**	**−0.84**	0.07
Firmicutes	*Enterococcaceae*	0.06	**1.00**	−0.14	**0.63**	0.33	**0.52**	0.07	**0.34**	−0.14	**0.26**	−0.01	**0.22**	0.03	0.17	0.07
	Erysipelotrichaceae	−0.04	−0.19	−0.10	−0.20	−0.06	−0.19	−0.08	−0.26	0.34	−0.02	0.17	−0.15	0.34	−0.26	0.49
	Lachnospiraceae	0.01	−0.25	−0.07	−0.18	−0.15	−0.25	0.02	0.19	0.03	0.24	−0.08	0.17	−0.01	0.12	0.08
	Lactobacillaceae	0.00	**2.13**	0.12	**1.92**	0.53	**1.71**	0.03	0.25	−0.12	0.08	−0.04	**0.57**	**−0.34**	**0.85**	−0.52
	Ruminococcaceae	0.01	−0.29	−0.05	−0.35	0.04	−0.36	−0.03	0.11	0.10	0.09	0.16	0.00	0.06	−0.14	0.02
	Streptococcaceae	0.13	**1.38**	−0.05	**1.28**	**0.29**	**1.28**	−0.09	**1.11**	**0.36**	**1.07**	0.07	**1.00**	**0.33**	**0.97**	0.29
	Veillonellaceae	0.08	0.08	0.05	0.25	−0.04	0.38	−0.03	0.70	0.02	0.62	0.18	0.67	0.09	0.60	0.05
Proteobacteria	*Burkholderiaceae*	0.07	0.39	−0.01	0.35	−0.07	0.43	−0.02	0.58	0.01	0.51	0.02	0.49	0.09	0.48	0.14
	Desulfovibrionaceae	0.02	−0.29	0.01	−0.15	−0.11	−0.08	0.11	−0.10	−0.07	−0.21	0.20	−0.18	0.04	−0.08	−0.15
	Enterobacteriaceae	−0.02	−0.20	**−0.24**	−0.21	**−0.26**	−0.07	**−0.31**	−0.21	**−0.21**	−0.21	−0.11	−0.16	**−0.21**	−0.14	−0.19
Verrucomicrobia	*Akkermansiaceae*	0.14	−0.10	−0.02	0.02	0.06	0.04	−0.06	−0.05	0.17	0.05	0.03	0.02	0.16	0.09	0.08

Table 5. Changes of microbial abundances at OTU level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of three human donors (C, D and F). The values represent average differences versus the respective reference (for fructans, the reference was the untreated blank, while for conditions with BB-12 addition this was the respective condition where BB-12 was not dosed) at the end of the colonic incubation over the three donors. For optimal visualization, increases due to treatment are highlighted with grey shading, with significant changes being indicated in bold (n = 9; 3 values for each of the 3 donors).

Phylum	Family	OTU#	Related Species	Blank	OF1		OF2		OF/IN1		OF/IN2		IN1		IN2		IN3	
				BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12	-	BB-12
Actinobacteria	*Bifidobacteriaceae*	2	*B. adolescentis*	−0.07	**1.15**	−0.24	**1.18**	**−0.26**	**0.99**	−0.13	**1.08**	**−0.18**	**1.01**	**−0.09**	**0.96**	**−0.08**	**0.98**	−0.08
		5	*B. animalis*	**2.19**	0.00	**2.99**	0.00	**2.82**	0.00	**2.85**	0.00	**2.81**	0.00	**2.64**	0.00	**2.72**	0.00	**2.73**
		21	*B. longum*	**−0.07**	**0.98**	**−0.32**	**1.06**	**−0.31**	**0.76**	**−0.32**	**0.51**	**−0.16**	**0.43**	−0.08	**0.48**	−0.14	**0.48**	**−0.19**
	Coriobacteriaceae	20	*Collinsella aerofaciens*	−0.10	0.14	0.32	0.16	0.20	0.29	0.10	0.15	0.07	−0.02	**0.26**	−0.03	**0.28**	0.16	0.21
	Eggerthellaceae	4	*Senegalimassilia sp.*	0.01	−0.01	**0.15**	0.01	0.03	0.07	**0.13**	**0.34**	**−0.07**	**0.24**	0.01	**0.24**	0.04	0.29	0.03
		7	*Ellagibacter isourolithinifaciens*	0.04	**0.71**	0.23	**0.72**	0.22	**0.96**	0.00	**0.78**	0.06	**0.89**	−0.05	**1.01**	−0.11	**0.91**	0.13
Bacteroidetes	*Bacteroidaceae*	9	*Bacteroides caccae*	−0.02	**−0.40**	0.04	**−0.48**	0.15	**−0.27**	**0.12**	**0.81**	0.09	**1.00**	−0.01	**0.75**	0.01	**0.46**	0.20
		6	*Bacteroides dorei*	−0.05	**−0.95**	0.07	**−1.02**	0.17	**−0.60**	0.03	**−0.60**	0.16	**−0.53**	−0.01	**−0.46**	0.07	**−0.46**	0.08
		11	*B.uniformis*	−0.03	**−0.72**	0.01	**−0.71**	0.02	**−0.71**	0.09	0.07	−0.04	0.12	−0.03	−0.11	−0.01	−0.17	−0.05
	Rikenellaceae	13	*Alistipes onderdonkii*	−0.08	0.63	0.21	1.10	−0.14	0.71	−0.21	0.16	0.06	0.13	0.04	0.22	−0.05	0.28	−0.04
Firmicutes	*Lactobacillaceae*	19	*Lactobacillus fermentum*	0.00	**1.94**	0.10	**1.76**	0.56	**1.59**	0.00	0.18	−0.08	0.02	−0.02	**0.47**	**−0.28**	**0.81**	−0.51
	Lachnospiraceae	10	*Blautia faecis*	0.11	−0.31	0.00	−0.28	−0.03	−0.05	−0.20	**0.45**	0.05	**0.68**	−0.15	**0.55**	−0.12	**0.57**	−0.07
		23	*butyrate−producing SR1/5*	0.01	0.02	0.01	0.08	−0.03	0.20	−0.13	0.22	0.04	0.25	−0.01	0.30	0.00	0.23	−0.04
		16	*Dorea longicatena*	−0.14	−0.26	0.05	−0.41	0.08	−0.30	0.05	−0.23	0.14	−0.16	−0.03	−0.30	0.08	−0.23	0.01
		17	*Clostridium bolteae/clostridioforme*	−0.06	−0.52	0.09	−0.81	−0.02	−0.09	0.05	0.28	0.00	0.21	0.06	0.24	−0.03	0.26	0.01
	Ruminococcaceae	12	*Faecalibacterium prausnitzii*	0.06	**−0.45**	0.04	**−0.43**	−0.03	**−0.43**	0.02	−0.23	0.31	**−0.10**	**0.14**	**−0.31**	0.06	**−0.45**	**0.22**
		8	*Faecalibacterium prausnitzii*	0.01	0.25	−0.24	0.29	−0.06	0.29	−0.02	**1.28**	0.05	**1.23**	0.24	**1.09**	0.25	**0.82**	−0.04
	Veillonellaceae	18	*Dialister succinatiphilus*	0.06	0.21	0.05	0.33	−0.07	0.48	−0.06	0.62	0.02	0.57	0.11	0.62	0.01	0.61	0.00
Proteobacteria	*Burkholderiaceae*	3	*Sutterella wadsworthensis*	0.06	**0.47**	−0.13	0.32	0.05	**0.47**	0.05	**1.07**	−0.02	**1.03**	−0.02	**0.99**	0.01	**0.77**	0.29
	Enterobacteriaceae	1	*Escherichia coli*	−0.02	−0.20	**−0.24**	−0.21	**−0.26**	−0.07	**−0.31**	−0.21	**−0.21**	−0.21	−0.11	−0.16	**−0.21**	−0.14	−0.19

All fructans consistently increased Actinobacteria numbers, with an additional stimulatory effect being observed upon co-administration of BB-12. At family level, increased Actinobacteria numbers were mainly attributed to significant stimulation of *Bifidobacteriaceae*. At the lowest phylogentic level, it was observed that all fructans boosted the growth of *Bifidobacteriaceae* OTU2 (related to *Bifidobacterium adolescentis*) and OTU21 (related to *Bifidobacterium longum*), while administration of BB-12 specifically enhanced engraftment of *Bifidobacterium animalis* OTU5. Upon the increase of BB-12, *Bifidobacteriaceae* OTU2 and OTU21 tended to decrease, suggesting competition with BB-12. All fructans also significantly increased levels of *Eggerthellaceae* OTU7 (related to *Ellagibacter isourolithinifaciens*) within the Actinobacteria phylum.

Further, treatment with OF1, OF2 and OF/IN1 significantly reduced Bacteroidetes numbers, which was attributed to reduction of *Bacteroidaceae* levels. Co-supplementation of BB-12 with OF/IN2 on the other hand significantly stimulated *Bacteroidaceae* numbers. At OTU level, *Bacteroidaceae* OTU9 (related to *Bacteroides caccae*) increased upon supplementation of the longer fructans (OF/IN2, IN1, IN2 and IN3), while specifically decreasing for the shorter fructans (OF1, OF2 and OF/IN1). Additionally, *Bacteroidaceae* OTU11 (related to *Bacteroides uniformis*) specifically decreased upon treatment with OF1, OF2 and OF/IN1, while *Bacteroidaceae* OTU6 (related to *Bacteroides dorei*) strongly decreased for all fructans. Furthermore, all fructan-type carbohydrates decreased *Tannerellaceae* numbers significantly, while combination of OF/IN2 and IN2 with BB-12 again allowed stimulated this group.

With respect to Firmicutes numbers, enhanced levels were observed upon treatment with OF2, OF/IN2 and IN1, while co-administration of OF/IN2 with BB-12 even further increased Firmicutes numbers. Overall, all fructans increased *Enterococcaceae* (except IN3), *Streptococcaceae* and *Lactobacillaceae* (except OF/IN2 and IN1) levels. *Streptococcaceae* abundance even increased further upon supplementation of BB-12 in combination with OF2, OF/IN2 and IN2. *Lactobacillaceae* levels were most strongly increased for the shorter fructans (OF1, OF2 and OF/IN1), which was attributed to stimulation of *Lactobacillaceae* OTU9 (related to *Lactobacillus fermentum*). On the other hand, especially longer fructans (OF/IN2, IN1, IN2 and IN3) significantly increased levels of the butyrate-producing *Lachnospiraceae* OTU10 (related to *Blautia faecis*) and *Ruminococcaceae* OTU8 (related to *Faecalibacterium prausnitzii*).

Finally, no overall changes were observed in Proteobacteria and Verrucomicrobia upon treatment with the different test products. However, BB-12 decreased *Enterobacteriaceae* levels when co-administered with the fructan-type carbohydrates (except for IN1 and IN3), which was attributed to reduced levels of *Enterobacteriaceae* OTU1 (related to *Escherichia coli*).

4. Discussion

This study evaluated combinations of *Bifidobacterium animalis* subsp. *lactis* BB-12 with seven fructans ranging from oligofructose (OF1-OF2; low degree of polymerization (DP)), mixtures of oligofructose/inulin (OF/IN1-OF/IN2) up to inulin (IN1-IN2-IN3; high DP), using a combination of monoculture incubations and short-term colonic incubations incorporating a simulation of the complex human colon microbiota. The conducted experiments not only revealed a marked modulation of microbial activity and composition upon fructan administration but it also pointed out several additional effects due to co-administration of BB-12.

First, monoculture experiments revealed that the fermentation of fructans by BB-12 was substrate-dependent, with BB-12 most strongly fermenting fructans with low DP (OF1 and OF2). This followed from the strong growth of BB-12 and strong increase in acetate/lactate levels for OF1 and OF2. Other studies have indeed already demonstrated that fructan fermentation by BB-12 depends on the molecular structure of the fructan [20,36,37], with enhanced growth of BB-12 for fructans with low DP [20,38,39]. Furthermore, inability of BB-12 to metabolize long-chain inulins, has been observed before as well [37,38,40]. Overall, these monoculture-derived data confirmed the potential of developing synbiotic

combinations between BB-12 and the tested fructan-type carbohydrates, where specifically selected fructans would support the growth of BB-12.

A peculiar finding was that, in presence of a complex microbiota, fructan administration boosted the growth of BB-12 for all fructans tested across all three donors (as measured via abundance of OTU5 that was exclusively detected when BB-12 was dosed). While being most pronounced for OF1, this effect was also observed for fructans with high DP that were hardly fermented by BB-12 in monoculture, suggesting that BB-12 has high potential to compete with the human colon microbiota when fructans are present, independently of the molecular structure of the fructan. As a potential explanation, when focusing on the degradation of raftiline, Mueller et al. [20] observed preferential fermentation of shorter fructan fractions. Such fermentation pattern has previously been linked with intracellular fructan fermentation [41,42]. By importing short oligofructose fractions, BB-12 could thus have a competitive advantage as opposed to other lactic acid bacteria that ferment fructans via extracellular mechanisms by which they have to import fructose that could meanwhile already have been fermented by other gut microbes [43]. While during monoculture experiments, such oligosaccharides were already present in some test products as such (OF1-OF2 and to a lesser extent OF/IN1-OF/IN2), in presence of a mixed microbiota, members of the indigenous microbiota could release oligosaccharides from long fructans (IN1-IN2-IN3), which BB-12 could then ferment intracellularly. This could explain the observed competitiveness of BB-12 to grow within the complex colon microbiota in presence of fructans. As a side remark, the fitness of BB-12 also followed from the donor screening (experiment 2), where BB-12 was capable to grow on starch in presence of sterile fecal suspensions of all six donors tested as followed from increased *Bifidobacterium* numbers and a reduction in pH due to increased acetate/lactate levels.

Prior to elaborating on the effects of synbiotic administration on the microbial activity and composition, it has to be noted that treatment with the fructan-type carbohydrates as such already exerted strong effects. This involved a markedly decreased pH due to increased production of acetate and lactate, while proteolytic fermentation was lowered. At community level, the increased production of acidic end-metabolites was associated with a strong bifidogenic effect, mainly attributed to stimulation of *Bifidobacteriaceae* OTUs related to *Bifidobacterium adolescentis* and *Bifidobacterium longum*. Strong bifidogenic effects have indeed been linked with consumption of fructan-type carbohydrates in both in vitro and in vivo studies (as reviewed by Kolida et al. [44]). *Bifidobacterium* species are well-documented primary substrate degraders, able to produce high amounts of acetate and lactate [45,46]. Several health-promoting properties have been associated with bifidobacteria, including the protection against pathogenic infection in the gastrointestinal tract [47]. Furthermore, both *Bifidobacterium longum* and *Bifidobacterium adolescentis* strains have been correlated with immunomodulatory responses, including reduction of inflammation and supporting intestinal epithelial barrier integrity [48–50]. Next to strong stimulation of *Bifidobacteriaceae* numbers, fermentation of fructan-type carbohydrates resulted in stimulation of the lactate-producing *Enterococcaceae*, *Streptococcaceae* and *Lactobacillaceae* families. *Lactobacillaceae* levels were most strongly increased for the fructans categorized by a low DP (OF1, OF2 and OF/IN1), resulting in a more pronounced stimulation of lactate levels compared to the fructans with a higher DP. Fermentation of the fructan-type carbohydrates characterized by a high DP on the other hand more strongly stimulated propionate and butyrate levels, indicating a product-dependent microbial response. Indeed, Rossi et al. [51] have shown a different SCFA profile upon supplementing oligofructose and inulin in vitro. While the major end-metabolites upon fermentation of oligofructose were acetate and lactate, fermentation of long-chain inulin resulted in high concentrations of butyrate. In the present study, increased butyrate levels were mainly attributed to the enhancement of OTUs related to the *Blautia faecis* and *Faecalibacterium prausnitzii*. *Blautia* species have been shown to exert a key role in fermentation of carbohydrates [52] and are therefore often associated with a healthy gut microbiome. Furthermore, anti-inflammatory effects have been attributed to the *Blautia* genus [53]. Similarly, *Faecalibacterium prausnitzii* is a

potent butyrate-producing bacterium with well-documented anti-inflammatory properties [54]. Like *Blautia*, this bacterial species has been reported to be reduced in the gut microbiome of patients suffering from intestinal disorders, including colorectal cancer [55]. Overall, the strong bifidogenic effect as well as stimulation of other health-related bacterial species stresses the potent prebiotic potential of fructans, with differences in metabolic response related with the molecular structure of fructans, stressing the importance of proper selection.

While co-administration of BB-12 with the different fructan-type carbohydrates resulted in similar metabolic effects compared to fructan supplementation, some additional changes (e.g., increased acetate, propionate and butyrate levels) were observed for specific synbiotic combinations, indicating that BB-12 was involved in the fermentation process. Furthermore, when co-administered with fructans, BB-12 decreased *Enterobacteriaceae* numbers, a family known to contain several opportunistic pathogenic species [56], a decrease that reached statistical significance for most fructans tested. Overall, the observed metabolic changes and the successful engraftment of the probiotic strain in presence of the prebiotic fructan-type carbohydrates confirm the synergistic effect of co-administration of BB-12.

As a remark, during a first series of non-reported experiments, in which no background sterile fecal suspension was co-administered, none of the fructans were fermented by BB-12. However, when the test was repeated in presence of sterile fecal suspension, each of the fructan-type carbohydrates were fermented by the BB-12 strain in a product-specific fashion, indicating that essential co-factors were likely present in the sterile fecal suspensions enabling metabolic activity of BB-12. Overall, this demonstrated the importance of supplementing essential co-factors when investigating fermentation of substrates in monocultures.

In conclusion, the combination of in vitro gut models with a novel technique for quantitative determination of microbiome composition allowed to reveal the synbiotic potential of fructan-type carbohydrates combined with BB-12. While a strong effect on microbial activity and composition was demonstrated for fructan administration as such, co-administration of BB-12 resulted in additional effects including more profound bifidogenic effects, additional production of health-related metabolites and suppression of opportunistic pathogenic organisms, altogether stressing the potential of synbiotics consisting of fructans with BB-12. While fructans with low DP seem most promising as BB-12 can ferment them optimally in monoculture, also fructans with higher DP boosted the selective growth of BB-12 in presence of a mixed microbiota, which was likely due to the release of oligosaccharides from inulin. As a final remark, *in vivo*, synbiotic supplements are generally administered over the course of several days or weeks. Future research could therefore focus on the effects of repeated administration of the synbiotic formulations to further elucidate the potential synergistic and stimulatory effects on BB-12 selective growth, health-related microbial metabolite production and changes in intestinal microbiome composition. Furthermore, inclusion of the study of host-microbiome interactions could provide further insight in the potential immune modulation of the synbiotic formulations related with health benefits [57].

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/2/598/s1, Figure S1: Metabolic activity of BB-12 in monoculture in presence of sterile fecal suspension of six donors (A, B, C, D, E and F) upon dosing starch as carbon source, Figure S2: Metabolic activity of fecal microbiota of the six same donors as such (A, B, C, D, E and F) upon dosing starch as carbon source, Table S1: Changes in metabolic activity upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of three human donors (C, D and F), Table S2: Changes of microbial abundances at phylum level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of three human donors (C, D and F), Table S3: Changes of microbial abundances at family level (log(cells/mL)) as detected via quantitative 16S-targeted

Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of donor C, Table S4: Changes of microbial abundances at family level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of donor D, Table S5: Changes of microbial abundances at family level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of donor F, Table S6: Changes of microbial abundances at OTU level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of donor C, Table S7: Changes of microbial abundances at family level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of donor D, Changes of microbial abundances at family level (log(cells/mL)) as detected via quantitative 16S-targeted Illumina sequencing at the end of the colonic incubation (48 h) upon dosing BB-12 and/or seven fructans with short (OF1, OF2), intermediate (OF/IN1, OF/IN2) and high degree of polymerization (IN1, IN2 and IN3) to the complex microbiota of donor F.

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